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Effects of Sterol Biosynthesis Inhibitor Fungicides in the Phytopathogenic Fungus, *Nectria haematococca:* Ergosterol Depletion versus Precursor or Abnormal Sterol Accumulation as the Mechanism of Fungitoxicity

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Abstract: The relative importance of the depletion of ergosterol versus the accumulation of precursor or abnormal sterols in the mechanism of fungal growth inhibition by sterol biosynthesis inhibitor fungicides is incompletely understood. In order to investigate this problem further, the degree of inhibition of the growth of *Nectria haematococca* by fungicides with different enzymatic targets in the sterol biosynthetic pathway was determined and compared with their effects on the sterol profile. The sensitivity of *N. haematococca* was highest towards fenpropimorph, followed by tebuconazole, terbinafine, fenpropidin and tridemorph. Terbinafine, a squalene epoxidase inhibitor, induced a very large accumulation of squalene without very significant inhibition of ergosterol biosynthesis and growth. The fungus appeared able to tolerate large amounts of squalene. In the case of tebuconazole, a sterol 14α-demethylase inhibitor, it seemed that the accumulation of C4 mono- and dimethyl sterols was responsible for fungitoxicity. Fenpropimorph and fenpropidin seemed to be good inhibitors of both sterol Δ^{14}-reductase and $\Delta^{8} \rightarrow \Delta^{7}$-isomerase, whereas tridemorph was a better inhibitor of $\Delta^{8} \rightarrow \Delta^{7}$-isomerase than of the Δ^{14}-reductase. Large accumulations of $\Delta^{8,14}$- or Δ^{8}-sterols and correspondingly large decreases in the ergosterol content are both implicated in the fungitoxicity of these compounds in *N. haematococca.* © 1998 Society of Chemical Industry

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Key words: fenpropidin; fenpropimorph; fungitoxicity; *Nectria haematococca;* sterols; tebuconazole; terbinafine; tridemorph

NOMENCLATURE

Abbreviations and nomenclature throughout this paper are as follows: SBI = sterol biosynthesis inhibitor; DMI = 14α-demethylation inhibitor; sterol trivial and systematic names: all sterols referred to in the text have the 5α-configuration; eburicol, 4,4,14α-trimethylergosta-8,24(24^1)-dien-3β-ol; 4,4-dimethylfecosterol, 4,4-dimethylergosta-8,24(24^1)-dien-3β-ol; 4α-methylfecosterol, 4α-methylergosta-8,24(24^1)-dien-3β-ol; ergosterol, ergosta-5,7,22E-trien-3β-ol; episterol, ergosta-7,24(24^1)-dien-3β-ol; fecosterol, ergosta-8,24(24^1)-dien-3β-ol; ignosterol, ergosta-8,14-dien-3β-ol; lanosterol, 4,4,14α-trimethylcholesta-8,24(25)-dien-3β-ol; lichesterol, ergosta-5,8,22E-trien-3β-ol; obtusifoliol, 4α,14α-dimethylergosta-8,24(24^1)-dien-3β-ol.

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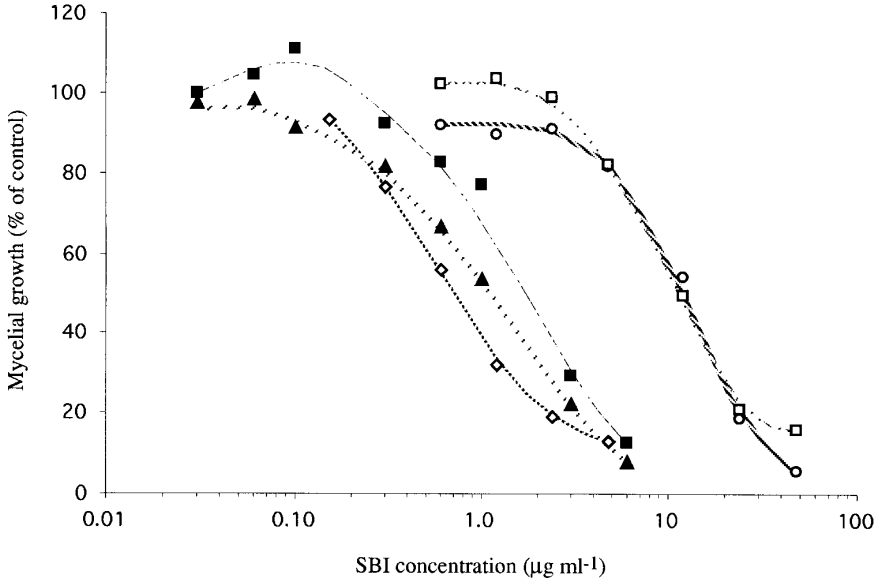

Fig. 1. Effect of SBIs on mycelial growth of *Nectria haematococca* in liquid medium: (■) terbinafine, (▲) tebuconazole, (◇) fenpropimorph, (□) fenpropidin and (○) tridemorph.

1 INTRODUCTION

Sterol biosynthesis inhibitors (SBIs) are largely used as antimycotics and agricultural fungicides.[1,2] To date, allylamines (e.g. terbinafine), which are squalene epoxidase inhibitors,[3] have been developed only for medicinal use against fungi. Two groups of SBIs are commonly used in agriculture against phytopathogenic filamentous fungi. The largest group includes pyridines, pyrimidines, piperazines and azoles (e.g. tebuconazole), all of which are sterol 14α-demethylation inhibitors (DMIs).[2] Azoles have also been developed and intensively used against mammalian fungal pathogens.[1] Morpholines (e.g. fenpropimorph, tridemorph) and piperidines (e.g. fenpropidin), which constitute the second group, are inhibitors of the sterol Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase.[2] SBIs lead to a decrease in the amount of the major sterol and the accumulation of precursor or abnormal sterols.[2] The main final sterol of most filamentous fungi is ergosterol.[4,5] SBI treatment induces accumulations of squalene, 14α-methylsterols or $\Delta^{8,14}$- and Δ^8-sterols in the cases of allylamines, DMIs or morpholines/piperidines, respectively. Sterols are essential for the cell and have several different functions, including a bulk membrane role,[6] for which large amounts of sterols are needed, and involvement in cell proliferation, (called either sparking,[7] synergistic,[8] hormonal[6] or sparing[9] function) for which the quantities needed appear to be much smaller.[10,11] The structural requirements for sterols to fulfil the bulk membrane function appear to be broad but those for the cell proliferation role appear to be highly specific.[10,11] The mechanism of SBI fungitoxicity is not completely understood. Some authors suggest that the accumulation of abnormal or precursor sterols is responsible for toxicity, whereas others place greater emphasis on the reduction of ergosterol content.[11–13] We have studied the effects on mycelial growth and on sterol composition of several SBIs with different modes of action in order to obtain further indications on the mechanisms of their fungitoxicity. *Nectria haematococca*, an ascomycete fungal pathogen of pea, which has already been the subject of extensive genetic and biochemical studies in our laboratory, was chosen as a model.[14,15]

2 EXPERIMENTAL

2.1 Fungal strain

Nectria haematococca Berk. and Br., S1, perfect form of *Fusarium solani* f. sp. *pisi* was kindly provided by Prof. Van Etten.[16] The sensitivity of *N. haematococca* towards terbinafine, tebuconazole, fenpropimorph, fenpropidin and tridemorph, when grown on potato-dextrose agar medium, had been obtained previously; the EC_{50} values (effective concentrations causing a 50% reduction in the mycelial growth) were 0·2, 1·2, 1·7, 2·9 and 5·1 µg ml^{-1} for fenpropimorph, tebuconazole, fenpropidin, terbinafine and tridemorph, respectively.[14,15]

2.2 Liquid culture method

Conidia were incubated (10^5 conidia ml^{-1}) in liquid nutrient medium (KH_2PO_4 3·0, $MgSO_4$ 7 H_2O 0·3, $NaNO_3$ 3·0, glucose 8·0 and yeast extract 2·0 g $litre^{-1}$) in the absence or presence of fungicide, at 25°C under agitation at 150 rev min^{-1} on a rotary shaker for 20 h in order to obtain mycelium in exponential growth phase. The fungicides were added to the culture medium in ethanolic solutions (final concentration 5·0 ml $litre^{-1}$ ethanol), simultaneously with conidia. The mycelium

TABLE 1
Sterol and Squalene Contents in *Nectria haematococca* Cultivated in the Absence or Presence of Terbinafine, Tebuconazole, Fenpropimorph, Fenpropidin or Tridemorph

		SBI[a]				
		Terbinafine	*Tebuconazole*	*Fenpropimorph*	*Fenpropidin*	*Tridemorph*
Lipids	*Control*	*1 (35)*	*0·6 (35)*	*0·3 (25)*	*2·4 (5)*	*1·2 (10)*
Sterols[b]						
4,4-Dimethylsterols						
Lanosterol	0·5	0·3	0·3	0·2	0·2	0·1
Eburicol	1·1	0·6	41·9	0·5	1·2	0·9
4,4-dimethylergosta-8,14,24(24)[1]-trien-3β-ol	—[c]	—	—	Tr[d]	NS[e]	—
4,4-dimethylfecosterol	0·9	0·2	—	0·3	0·6	1·1
4α-Methylsterols						
Obtusifoliol	—	—	13·8	—	—	—
4α-methylergosta-8,14,24(24)[1]-trien-3β-ol	Tr	0·1	—	Tr	Tr	Tr
4α-methylfecosterol	1·0	0·4	—	0·5	0·5	0·9
4-Desmethylsterols						
Ergostatetraen-3β-ol	0·9	NS	0·4	NS	NS	1·3
Ergosta-5,8,14,22-tetraen-3β-ol	—	—	—	3·5	3·2	—
Lichesterol	1·3	1·7	1·7	3·4	4·8	25·8
Unknown sterol[f]	—	—	0·8	—	2·3	2·5
Ergosterol	91·1	95·3	40·4	37·8	45·8	43·5
Ergosta-8,14,24(24)[1]-trien-3β-ol	—	—	—	9·2	5·1	0·7
Ignosterol	—	—	—	30·6	17·2	0·4
Fecosterol	0·7	—	—	2·5	2·4	14·4
Ergost-8-en-3β-ol and unknown sterol[g]	—	—	—	4·1	3·1	6·7[h]
Ergosta-5,7-dien-3β-ol	Tr	—	0·1	—	—	—
Episterol	2·1	1·4	0·4	5·1	8·3	1·2
Ergost-7-en-3β-ol	—	—	—	1·9	4·9	—
Other sterols[i]	0·4	0·0	0·2	0·4	0·4	0·5
4,4-Dimethylsterols	2·5	1·1	42·2	1·0	2·0	2·2
4α-Methylsterols	1·0	0·5	13·8	0·5	0·5	0·9
4-Desmethylsterols	96·5	98·4	44·0	98·5	97·5	96·9
Total sterol amount (μg mg^{-1} dry weight)	4·5	3·0	4·7	6·1	3·3	4·3
Squalene (μg mg^{-1} dry weight)	0·03	15·5	ND[j]	0·02	0·01	0·002

[a] Expressed as μg ml^{-1}, percentage growth inhibition in parentheses.
[b] Expressed as percentage of total sterols.
[c] —: not detected.
[d] TR: trace (below 0·1% of total sterols).
[e] NS: not separately integrated because of the low level compared to the sterol with a very close RR_t value.
[f] Unknown sterol with an RR_t value between those of lichesterol and ergosterol.
[g] Ergost-8-en-3β-ol, probably, in mixture with an unknown sterol.
[h] Ergost-8-en-3β-ol, only.
[i] Sum of sterols (4,4-dimethyl, 4α-methyl or 4-desmethyl sterols) in very low amount (each of them below 0·1% of total sterols), or unidentified.
[j] ND: not done.

harvested was filtered on a 125 μm-mesh gauze disc, washed with an isotonic solution, frozen and kept at -80°C. The frozen mycelium was then lyophilised and weighed in order to calculate the EC_{50} in liquid culture, inhibition of growth being expressed as the percentage of dry weight, relative to untreated control cultures.

2.3 Squalene and sterol extraction and analysis

The lyophilised mycelium was saponified in methanolic potassium hydroxide (60 g litre^{-1}) at 70°C for 2 h. The extraction and purification of squalene and sterols (4,4-dimethyl, 4α-methyl and 4-desmethyl sterols) have been

TABLE 2
Sterol and Squalene Content in *Nectria haematococca* Cultivated in the Absence or the Presence of Fenpropimorph

		Fenpropimorph concentration (μg ml^{-1})					
	Control	*0·03*	*0·15*	*0·3*	*0·6*	*1·2*	*2·4*
4,4-Dimethylsterols[a]	2·5	ND[b]	1·7	1·0	1·0	0·6	0·5
4α-Methylsterols	1·0	ND	0·6	0·5	0·4	0·3	0·2
4-Desmethylsterols	96·5	100[c]	97·7	98·5	98·6	99·1	99·3
Ergosterol	91·1	84·1	50·5	37·8	15·7	6·8	5·2
$\Delta^{8,14}$-sterols	—[d]	6·5	39·4	43·3	59·5	78·3	87·6
Ergosta-5,8,14,22-tetraen-3β-ol	—	0·4	3·6	3·5	3·9	4·8	5·3
Ergosta-8,14,24(24)^1-trien-3β-ol	—	2·1	14·8	9·2	12·1	28·0	35·1
Ignosterol	—	4·0	21·0	30·6	43·5	45·5	47·2
Δ^8-sterols	2·0	6·0	5·7	10·0	11·1	7·8	4·0
Ergost-8-en-3β-ol[e]	—	1·3	0·6	4·1	5·9	3·0	2·4
Fecosterol	0·7	1·1	1·7	2·5	2·6	3·0	NS[f]
Lichesterol	1·3	3·6	3·4	3·4	2·6	1·8	1·6
Δ^7-sterols	2·1	2·2	1·3	7·0	10·9	4·6	0·8
Ergost-7-en-3β-ol	—	0·2	0·2	1·9	3·4	0·7	—
Episterol	2·1	2·0	1·1	5·1	7·5	3·9	0·8
Other 4-desmethylsterols	1·3	1·2	0·8	0·4	1·4	1·6	1·7
Total sterol amount (μg mg^{-1} dry weight)	4·5	4·9	3·4	6·1	6·5	7·7	9·3
Squalene[g]	100	ND	ND	67	67	100	167

[a] Expressed as % of total sterols.
[b] ND: not done.
[c] Expressed as % of 4-desmethylsterols.
[d] —: not detectable.
[e] Overestimated because ergost-8-en-3β-ol in mixture with an unidentified sterol.
[f] NS: not separately integrated because of the very low level of fecosterol relative to ignosterol, integrated with ignosterol.
[g] Expressed as % of control amount.

described elsewhere.[17] The sterol fractions were acetylated. The squalene and the steryl acetates were then analysed by GC with a flame ionisation detector and an OV-1 glass capillary column 30 m × 0·32 mm, carrier gas N_2 0·5 bar, oven temperature 250°C for squalene analyses and 300°C for steryl acetate analyses. Cholesterol was used as a standard for RR_t (relative retention time) determination and sterol quantification. Further analyses by GC/MS were performed with a Ribermag R10-10-C spectrometer. The GC was equipped with a CPSIL5CB column and the oven temperature programmed from 250°C to 320°C at 5°C min^{-1}. The ionising potential was 70 eV. The steryl acetates were identified from their RR_t values and their mass spectra, which are described elsewhere.[17,18]

3 RESULTS

3.1 Sensitivity of *Nectria haematococca* towards SBIs

The sensitivity of *N. haematococca* towards SBI fungicides, when cultivated in liquid medium, was highest towards fenpropimorph, followed by tebuconazole and terbinafine. The lowest sensitivities were obtained with fenpropidin and tridemorph (Fig. 1). The deduced EC_{50} values were 0·7, 1·1, 1·7, 11·4 and 11·6 μg ml^{-1} for fenpropimorph, tebuconazole, terbinafine, fenpropidin and tridemorph, respectively.

3.2 Effect of SBIs on squalene and sterol content

Ergosterol was the major sterol in the untreated cultures. It represented 91% of total sterols (Table 1). In Table 1 are also presented the detailed sterol profiles of *N. haematococca* cultivated in presence of SBIs at concentrations leading, except for terbinafine, to an ergosterol content of 38 to 46%, which corresponded to decreases of 44 to 63% in ergosterol content per mg dry weight, relative to the amount in untreated cultures. These SBI concentrations induced growth inhibition of 35% for terbinafine and tebuconazole, 25% for fenpropimorph, 5% for fenpropidin and 10% for tridemorph. In cultures treated with SBIs other than terbinafine, the ergosterol amounts decreased and precursors of ergosterol or abnormal sterols accumulated, but no modification of squalene content was detected.

TABLE 3
Sterol and Squalene Content in *Nectria haematococca* Cultivated in the Absence or the Presence of Fenpropidin

		Fenpropidin concentration (μg ml^{-1})					
	Control	*0·3*	*0·6*	*1·2*	*2·4*	*4·8*	*12*
4,4-Dimethylsterols[a]	2·5	2·5	2·3	1·2	2·0	0·9	0·9
4α-Methylsterols	1·0	1·0	1·1	0·6	0·5	0·2	0·2
4-Desmethylsterols	96·5	96·5	96·6	98·2	97·5	98·9	98·9
Ergosterol	91·1	80·9	80·2	68·9	45·8	54·7	32·3
$\Delta^{8,14}$-sterols	—[b]	0·6	1·9	13·0	25·5	17·2	40·7
Ergosta-5,8,14,22-tetraen-3β-ol	—	—	—	2·0	3·2	2·2	5·5
Ergosta-8,14,24(24)^1-trien-3β-ol	—	0·5	0·4	2·3	5·1	4·3	9·4
Ignosterol	—	0·1	1·5	8·7	17·2	10·7	25·8
Δ^8-sterols[c]	2·0	3·7	3·3	7·0	10·3	11·5	10·7
Ergost-8-en-3β-ol[c]	—	0·2	0·6	1·4	3·1	3·5	2·5
Fecosterol	0·7	Tr[d]	0·5	1·3	2·4	3·0	3·6
Lichesterol	1·3	3·7	2·2	4·3	4·8	5·0	4·6
Δ^7-sterols	2·1	5·4	8·7	6·7	13·2	10·9	13·1
Ergost-7-en-3β-ol	—	1·5	2·9	2·6	4·9	3·9	4·7
Episterol	2·1	3·9	5·8	4·1	8·3	7·0	8·4
Other sterols	1·3	5·9	2·5	2·6	2·7	4·6	2·1
Total sterol amount (μg mg^{-1} dry weight)	4·5	3·7	3·7	4·9	3·2	2·9	3·3
Squalene[e]	100	ND[f]	ND	96	122	ND	ND

[a] Expressed as % of total sterols.
[b] —: not detectable.
[c] Overestimated because ergost-8-en-3β-ol in mixture with an unidentified sterol.
[d] TR: trace (below 0·1% of total sterols).
[e] Expressed as % of control amount.
[f] ND: not done.

In the case of terbinafine treatment, although ergosterol increased slightly as a percentage of total sterols (the small increase, 91 to 95% of total sterols, is probably not significant), this corresponded to a decrease of 29% in ergosterol content per mg dry weight relative to the amount in untreated cultures. The total sterol amount per mg dry weight fell by 33%, but no abnormal sterols were detected and the relative amounts of the sterols present remained unchanged. The squalene content of cultures treated with 1 μg ml^{-1} terbinafine increased 500-fold.

In the case of tebuconazole treatment, 4-desmethyl sterols decreased whereas 4,4-dimethyl and 4α-methyl sterols increased. They represented 44, 42 and 14% of total sterols, respectively, compared with 96·5, 2·5 and 1% of total sterols in the control. These changes were due to the accumulation of the 14α-methylsterols di- and monomethylated at C4, eburicol and obtusifoliol, respectively. In treated cultures, eburicol, the sterol 14α-demethylase substrate, accumulated so that it was equivalent to the amount of ergosterol. Obtusifoliol, a 4,14α-dimethylsterol not detected in controls, was found in a significant amount (14%) in tebuconazole-treated cultures. However, no 4-desmethylsterol with a 14α-methyl group was detectable.

When the fungus was cultivated in the presence of fenpropimorph, fenpropidin or tridemorph, the proportions of 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols were not significantly different from those of fungus cultivated in absence of fungicide (Table 1), whatever the concentration used (Tables 2–4), although the composition within the three sterol classes was different. When *N. haematococca* was cultivated in presence of fenpropimorph or fenpropidin, mainly $\Delta^{8,14}$-sterols were accumulated, whereas treatment with tridemorph led to mainly Δ^8-sterols (Tables 2–4).

Among $\Delta^{8,14}$-sterols, 4,4-dimethylergosta-8,14,24(24^1)-trien-3β-ol and 4α-methylergosta-8,14,24(24^1)-trien-3β-ol, when detected at all, were found in very low amounts (below 0·5% of total sterols; data not shown) relative to $\Delta^{8,14}$-4-desmethylsterols. The major $\Delta^{8,14}$-sterols, in order of increasing quantities, were ergosta-5,8,14,22-tetraen-3β-ol, ergosta-8,14,24(24^1)-trien-3β-ol and ignosterol (Tables 2 and 3). They were found in this order at all fenpropimorph and fenpropidin concentrations tested and the amount of each of them increased with increasing fungicide concentration (e.g. at around EC_{50} values, for fenpropimorph, they represented 4, 12 and 44% of total sterols, respectively and for fenpropidin 6, 9 and 26% of total sterols, respectively). With

TABLE 4
Sterol and Squalene Content in *Nectria haematococca* Cultivated in the Absence or the Presence of Tridemorph

		Tridemorph concentration ($\mu g\ ml^{-1}$)				
	Control	*0·6*	*1·2*	*2·4*	*4·8*	*12*
4,4-Dimethylsterols[a]	2·5	3·5	2·2	4·6	3·0	2·4
4α-Methylsterols	1·0	1·3	0·9	1·0	1·8	1·4
4-Desmethylsterols	96·5	95·2	96·9	94·4	95·2	96·2
Ergosterol	91·1	52·6	43·5	22·6	24·9	23·4
$\Delta^{8,14}$-sterols	—[b]	0·7	1·1	3·5	2·5	5·4
Ergosta-5,8,14,22-tetraen-3β-ol	—	—	—	1·2	0·9	2·0
Ergosta-8,14,24(24)[1]-trien-3β-ol	—	0·4	0·7	0·2	0·9	1·8
Ignosterol	—	0·3	0·4	2·1	0·7	1·6
Δ^{8}-sterols	2·0	36·9	46·9	57·8	64·3	65·4
Ergost-8-en-3β-ol	—	5·4	6·7	10·1	5·4	4·0
Fecosterol	0·7	10·9	14·4	20·8	24·6	22·3
Lichesterol	1·3	20·6	25·8	26·9	34·3	39·1
Δ^{7}-sterols	2·1	1·4	1·2	1·7	0·7	0·9
Episterol	2·1	1·4	1·2	1·7	0·7	0·9
Other sterols	1·3	3·6	4·2	8·8	2·8	1·1
Total sterol amount ($\mu g\ mg^{-1}$ dry weight)	4·5	3·7	4·2	2·6	2·7	3·5
Squalene[c]	100	47	22	122	46	ND[d]

[a] Expressed as % of total sterols.
[b] —: not detectable.
[c] Expressed as % of control amount.
[d] ND: not done.

fenpropimorph and fenpropidin treatment, a slight Δ^8-sterol accumulation (ergost-8-en-3β-ol, fecosterol and lichesterol) was also detected (Tables 2 and 3). The Δ^8-sterol accumulation slightly increased with increasing concentration (Tables 2 and 3). As ergost-8-en-3β-ol did not separate from an unknown sterol, the Δ^8-sterol amount was probably slightly overestimated. A similar accumulation of Δ^7-sterols, episterol and ergost-7-en-3β-ol could be observed in the case of fenpropimorph and fenpropidin treatment. The amount of Δ^7-sterol accumulation was in the same order as that of Δ^8-sterols and also increased with increasing fungicide concentration (the maximum level was around 13% of total sterols). In the case of fenpropimorph, the percentage of Δ^8- and Δ^7-sterols then decreased at concentrations exceeding the EC_{50} value.

Among the main Δ^8-sterols which accumulated following tridemorph treatment, lichesterol was always present in higher amounts than fecosterol, whereas the amount of ergost-8-en-3β-ol was relatively low (e.g. at EC_{50} value, they represented 39, 22 and 4% of total sterols, respectively). Some $\Delta^{8,14}$-4-desmethylsterols were detected and the amount slightly increased with increasing tridemorph concentration, but did not exceed 5% of total sterols at the highest concentration used (EC_{50} value).

The total sterol content appeared relatively constant, varying from 3 to 4 $\mu g\ mg^{-1}$ dry weight, except in the case of fenpropimorph treatment where the amount increased, with increasing fungicide concentration, reaching 9 $\mu g\ mg^{-1}$ dry weight at 2·4 $\mu g\ ml^{-1}$ of fenpropimorph, at which concentration growth was inhibited by 90% (Tables 2–4).

Morpholine and piperidine treatments in *N. haematococca* had no effect on squalene contents, whatever the fungicide concentration used (Tables 2–4).

4 DISCUSSION

The fungitoxicity of SBIs in liquid medium was slightly different from that determined on agar medium,[14,15] especially for fenpropidin, where *N. haematococca* appeared seven times less sensitive in liquid medium than on agar medium. Fenpropidin, like fenpropimorph and tridemorph, can exist in neutral and protonated forms, the ratio of each form depending on the pK_a of the compound and the pH of the medium.[19] Fenpropimorph uptake studies have shown that uptake is dependent on pH, which can be explained by the pK_a value (data not shown). However, the lower sensitivity to fenpropidin in liquid medium could not be due to the pH

of the medium, as it was the same in potato-dextrose agar and liquid medium.

N. haematococca in liquid medium appeared sensitive towards terbinafine, tebuconazole and fenpropimorph and relatively tolerant towards fenpropidin and tridemorph. Growth inhibition was correlated with ergosterol biosynthesis inhibition, which led to ergosterol decrease and the accumulation of abnormal or precursor sterols, as described elsewhere.[1,2] However, no consistent relationship between growth inhibition and ergosterol decrease or abnormal or precursor sterol accumulation emerged. The effects of each SBI will therefore be discussed separately.

Terbinafine treatment led to squalene accumulation, indicating that squalene epoxidase was inhibited, as already described for several human fungal pathogens[20,21] and for the phytopathogenic fungus *Ustilago maydis* (DC) Corda.[22] No other target of terbinafine was detected in the sterol profile of *N. haematococca*. It was found in celery cell suspension cultures that Δ^{14}-reductase and especially Δ^{7}-reductase (an enzyme which does not participate in fungal ergosterol biosynthesis) could be secondary targets in plants.[23] In *N. haematococca*, although the ergosterol amount was only inhibited by 29%, squalene accumulated at a very high level (500 times higher in terbinafine-treated mycelium than in the control). To understand these results, it could be hypothesised that a high level of squalene might result in an increased rate of ergosterol biosynthesis, in the early steps, to compensate for squalene epoxidase inhibition. In fact, it has been postulated that squalene might regulate 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity, a critical regulatory enzyme in sterol biosynthesis.[11,24] Other SBIs tested in this study reduced ergosterol to a greater extent, whereas growth inhibition was either in the same order or less affected. It could thus be hypothesised that in *N. haematococca*, the very high level of squalene accumulation induced toxicity, rather than ergosterol decrease, as postulated by Ryder for *Candida* spp., *Trichophyton mentagrophytes* (Robin) Blanch and *Aspergillus fumigatus* Fres.[20,21] However, our results indicate that squalene itself is unlikely to be very toxic because *N. haematococca* could accumulate squalene in very large amounts without very significant growth inhibition and only moderate inhibition of ergosterol biosynthesis, which was not the case for the fungi mentioned in References 20 and 21. In the case of *U. maydis*, it has been hypothesised that the ergosterol decrease is responsible for growth inhibition.[22]

Tebuconazole treatment led to a decrease in the ergosterol level and accumulation of the 14α-methylsterols eburicol and obtusifoliol, indicating that tebuconazole inhibits sterol 14α-demethylation in *N. haematococca*, as described earlier in different experimental conditions[25] and as reported for other fungi[26,27] and for other DMIs.[2] No other 14α-methylsterols, such as 14α-methylfecosterol, found in several other fungi,[27–29] were detected in *N. haematococca*. Growth inhibition by tebuconazole was higher, with an ergosterol amount equally or less affected than with morpholines or piperidine. The accumulation of 14α-methylsterols mono- or dimethylated at C4 thus appears to contribute to fungitoxicity. It has been postulated that the axial 14α-methyl group could destroy the planarity of the α-face of the molecule, thus sterically impeding the ability of the sterol to stabilise membrane phospholipids and that this could explain DMI fungitoxicity.[30] However, there are examples showing that presence of 14α-methylsterols instead of normal sterols does not seem to lead to growth inhibition.[30] Studies on the structural requirements of sterols for membrane function allowing growth have also shown that the 14α-methyl group is not deleterious, whereas no sterol-like triterpenoid supports growth.[31] So, it could be hypothesised that tebuconazole fungitoxicity in *N. haematococca* might partly be due to 4α-methyl and 4,4-dimethyl sterol accumulation in large amounts.

Cultivation of *N. haematococca* in the presence of fenpropimorph, fenpropidin or tridemorph led to decreases in the amount of ergosterol and accumulations of $\Delta^{8,14}$- and Δ^{8}-sterols, mainly 4-desmethylsterols. These fungicides are thus very likely to be inhibitors of both Δ^{14}-reductase and $\Delta^{8} \rightarrow \Delta^{7}$-isomerase in *N. haematococca*, but to varying degrees.

Fenpropimorph, which led mainly to $\Delta^{8,14}$-sterol accumulation, even at low concentrations (at the lowest fenpropimorph concentration, with slight effects on ergosterol biosynthesis, the $\Delta^{8,14}$-sterol accumulation was greater than that of Δ^{8}-sterols) seems to be a potent inhibitor of Δ^{14}-reductase in *N. haematococca*, as published earlier[17,25] and as has been found in other fungi, such as *Penicillium italicum* Wehmer,[32] *Saccharomyces cerevisiae* Meyer ex Hansen,[33] and *N. haematococca* var. *cucurbitae*.[34] However, in the case of some other *Fusarium* species,[17] *U. maydis*,[35] *Botrytis cinerea* Pers.[12] (Debieu *et al.* unpublished), *Pseudocercosporella herpotrichoides*, (Fron) Deighton[12,36] and *Pyrenophora teres* Drechs.[12] it has been shown that both the Δ^{14}-reductase and the $\Delta^{8} \rightarrow \Delta^{7}$-isomerase were significantly inhibited.

For fenpropidin, the Δ^{14}-reductase also seems the main target, as mainly $\Delta^{8,14}$-sterols accumulated, as shown with *S. cerevisiae*,[33] *U. maydis*,[33,35] and *B. cinerea* (Debieu *et al.* unpublished). Only a slight Δ^{8}-sterol accumulation could be detected. Ziogas *et al.*[34] have shown that for fenpropidin, the inhibition of $\Delta^{8} \rightarrow \Delta^{7}$-isomerase in *N. haematococca* var. *cucurbitae* was more important than in our case. It could be noticed that, at the lowest fenpropidin concentration, our slight Δ^{8}-sterol accumulation was greater than that of $\Delta^{8,14}$-sterols.

It should be noted that Δ^{14}-reduction precedes $\Delta^{8} \rightarrow \Delta^{7}$-isomerisation in ergosterol biosynthesis. High

levels of inhibition of Δ^{14}-reduction could thus mask potential $\Delta^8 \rightarrow \Delta^7$-isomerisation inhibition. As the slight accumulation of Δ^8-sterols was evident at low fenpropimorph and fenpropidin concentrations, it could be argued that these fungicides are likely also to be potent inhibitors of the $\Delta^8 \rightarrow \Delta^7$-isomerase in *N. haematococca*. Enzymatic studies would be necessary to confirm this hypothesis. Such studies have been conducted in *S. cerevisiae*[37,38] and maize embryos,[39] (where it has been shown that, after fenpropimorph treatment, among abnormal phytosterols, plants accumulate also Δ^8- and $\Delta^{8,14}$-sterols),[40] and demonstrated that Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase were both good targets for fenpropimorph and fenpropidin.

Tridemorph led to mainly Δ^8-sterol accumulation in this study. $\Delta^{8,14}$-Sterols represented less than 6% of total sterols at the highest fungicide concentration. In the case of tridemorph, although Δ^8-sterols predominated, a higher $\Delta^{8,14}$-sterol amount has been reported earlier[25] (25% of total sterols) than in the present study. This could be due to differences in the experimental conditions (mycelium, instead of the conidia treatment used for this study and different fungicide incubation time). As $\Delta^8 \rightarrow \Delta^7$-isomerase occurs later than Δ^{14}-reductase in ergosterol biosynthesis and as Δ^8-sterols predominated over $\Delta^{8,14}$-sterols in the presence of tridemorph, it could be hypothesised that in *N. haematococca*, tridemorph is broadly more efficient as an inhibitor of the $\Delta^8 \rightarrow \Delta^7$-isomerase than the Δ^{14}-reductase. These results are in agreement with sterol profiles obtained in the presence of tridemorph with *S. cerevisiae*,[33] *U. maydis*,[33] *N. haematococca* var. *cucurbitae*,[34] *B. cinerea* (Debieu *et al.* unpublished) and with tridemorph concentrations leading to a 50% inhibition of Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase activity in *S. cerevisiae*[37] and maize embryos.[40]

The total amount of sterols was increased in the presence of fenpropimorph, as already observed in *S. cerevisiae*.[41] This overproduction may occur in response to a lack of feedback inhibition at 3-hydroxy-3-methylglutaryl coenzyme A reductase by the aberrant accumulated sterols, as suggested by Lorenz and Parks.[41] However, surprisingly, this increase was not obtained with fenpropidin nor with tridemorph treatment. A very slight Δ^7-sterol (episterol and ergost-7-en-3β-ol) accumulation could be detected in presence of fenpropimorph or fenpropidin. To explain this result, it could be postulated that fenpropimorph and fenpropidin may be able to inhibit Δ^5-desaturase or that Δ^7-sterol accumulation could be a consequence of the deregulation of ergosterol biosynthesis in the presence of these fungicides. Such a Δ^7-sterol accumulation has been described in the case of *Erysiphe graminis* DC f. sp. *hordei* Marchal, but in the presence of tridemorph, not in the presence of fenpropidin.[42] Moreover, it was much more marked than in our study and concerned episterol. The effects of morpholines and piperidine on Δ^7-sterol accumulation could be studied in other fungi to test these hypotheses. No squalene accumulation in *N. haematococca* was detected in the presence of fenpropimorph, fenpropidin and tridemorph, whatever the fungicide concentration. However, squalene accumulation has been reported in the presence of fenpropimorph in *N. haematococca* var. *cucurbitae*[34] and some other fungi belonging to the *Fusarium* genus[17] and, in the presence of fenpropidin, in *U. maydis*.[35]

Among morpholines and piperidine, *N. haematococca* appeared much more sensitive to fenpropimorph than to fenpropidin and tridemorph, in terms of both growth inhibition and ergosterol biosynthesis inhibition. Some authors have suggested that the fungicidal activity of fenpropimorph could be due to the production of ignosterol[12,41] which inserts into the membrane lipid bilayer, inducing hyperfluidity and leading to alterations in membrane behaviour, such as modified permeability, as shown by a decrease in the ability to accumulate glucose.[12] However, Marcireau *et al.*,[13,43] using sterol auxotroph mutants and gene disruption experiments concluded that Δ^{14}-reductase activity was necessary for *S. cerevisiae* growth. They suggested that ignosterol was efficient in membrane structure and that fenpropimorph fungitoxicity was due to ergosterol depletion and not ignosterol accumulation. Crowley *et al.*[44] isolated a Δ^{14}-reductase null mutant of *S. cerevisiae*, showing that ignosterol could be a suitable sterol for growth; however, growth was reduced compared to wild-type strain. However, they also showed that, under specific conditions, ignosterol may not be an appropriate sterol for growth. In *Neurospora crassa* Shear & Dodge, a Δ^{14}-reductase null mutant was also described with a reduced rate of growth and also reduced conidiation.[45,46] It could be hypothesised that $\Delta^{8,14}$-sterols might be suitable for growth but are not as efficient as ergosterol because of reduced growth and conidiation. So, in the presence of fenpropimorph, when the amounts of $\Delta^{8,14}$-sterols are very high and those of ergosterol are low, considerable growth inhibition would be expected. This is in agreement with our results, where fenpropimorph growth inhibition was correlated with ergosterol depletion and $\Delta^{8,14}$-sterol accumulation in *N. haematococca*, as clearly shown in Fig. 2.

Growth inhibition of *N. haematococca* by fenpropidin was detectable only from 2·4 $\mu g\ ml^{-1}$ and was very significant at 24 $\mu g\ ml^{-1}$. At the EC_{50} value, the percentage of $\Delta^{8,14}$-sterols (41% of total sterols) was higher than that of ergosterol (32% of total sterols) and could explain fungitoxicity, in agreement with our hypothesis postulating that $\Delta^{8,14}$-sterols are less efficient for growth than ergosterol.

In the case of tridemorph, the growth inhibition was marked from 2·4 $\mu g\ ml^{-1}$, where the percentage of ergosterol was 25% or lower, whereas Δ^8-sterols were above 50% of total sterols. Fungitoxicity could be due

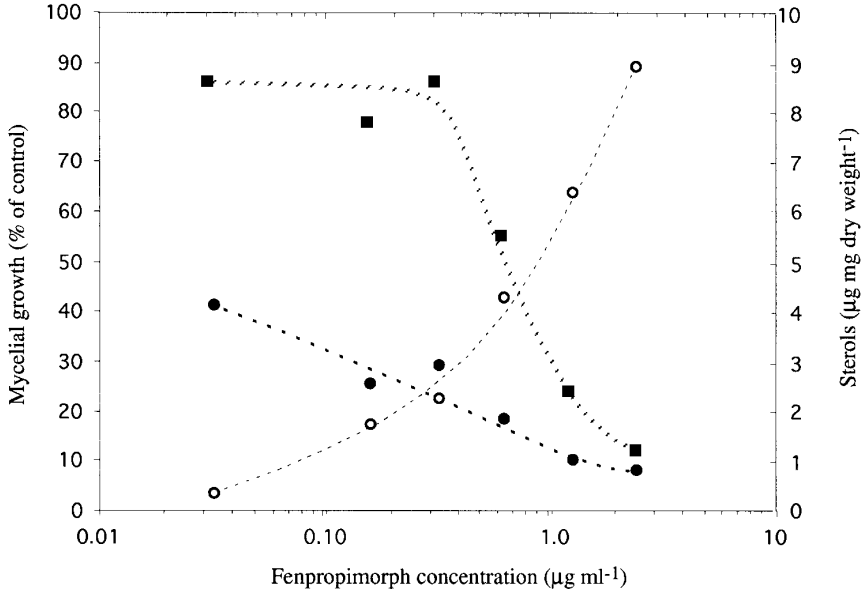

Fig. 2. Effect of fenpropimorph on (■) mycelial growth (●) ergosterol biosynthesis and (○) accumulation of $\Delta^{8,14}$-sterols in *Nectria haematococca*

to low ergosterol level or high Δ^8-sterol level. At low tridemorph concentrations, Δ^8-sterols were accumulated without significant growth inhibition. It thus seems that Δ^8-sterols may not be very toxic to *N. haematococca*. These results are in agreement with those obtained with a $\Delta^8 \rightarrow \Delta^7$-isomerase gene disruption study in *S. cerevisiae* showing that $\Delta^8 \rightarrow \Delta^7$-isomerase activity is not essential for yeast viability.[47] In addition, a $\Delta^8 \rightarrow \Delta^7$-isomerase-deficient strain of *N. crassa* has been selected.[45] However, as in the case of Δ^{14}-reductase deficiency, its growth and conidiation were reduced. So, as for $\Delta^{8,14}$-sterols, it could be hypothesised that Δ^8-sterols may be able to sustain growth, but not as efficiently as ergosterol.

Finally, it appears that neither $\Delta^{8,14}$-nor Δ^8-sterols are as efficient as ergosterol for growth and that they may be partly implicated in fungitoxicity. Studies among the *Fusarium* genus and *Pseudocercosporella herpotrichoides* fungi (actually named *Tapesia yallundae* for *P. herpotrichoides* var. *herpotrichoides* and *Tapesia acuformis* for *P. herpotrichoides* var. *acuformis*), have suggested that fenpropimorph toxicity could be related to inhibition of Δ^{14}-reductase rather than of $\Delta^8 \rightarrow \Delta^7$-isomerase.[17,36] In addition, studies have indicated that $\Delta^8 \rightarrow \Delta^7$-isomerase inhibition is not responsible for growth inhibition by fenpropimorph because of the absence of gene dosage effects, and ergosterol depletion and $\Delta^{8,14}$-sterol accumulation, rather than Δ^8-sterol accumulation was correlated with growth inhibition.[48] So, it could be asked if there may be a difference between $\Delta^{8,14}$- and Δ^8-sterols in their involvement in fungitoxicity in our present study. At fungicide concentrations near to EC_{50} values, the ratio between major abnormal sterols and ergosterol was 3·8 ($\Delta^{8,14}$), 1·3 ($\Delta^{8,14}$) and 2·8 (Δ^8) for fenpropimorph, fenpropidin and tridemorph, respectively. These values corresponded to ergosterol amounts of 1·0, 1·1 and 0·8 µg mg^{-1} dry weight, respectively, which represents an ergosterol decrease factor of about 4, relative to control. Thus abnormal sterol amounts reached values of 3·9, 1·3 and 2·3 µg mg^{-1} dry weight for fenpropimorph, fenpropidin and tridemorph, respectively. Because of the heterogeneity of the ratios of abnormal sterols and ergosterol at EC_{50} values, especially the difference between fenpropimorph and fenpropidin, it could be hypothesised that the fungitoxicity might be due to both large ergosterol decrease and large accumulations of $\Delta^{8,14}$- or Δ^8-sterols. Growth depends on both sterol bulk membrane and metabolic functions.[10,11] Metabolic functions need small amount of specific sterols, contrary to the bulk membrane function.[10,11] Under our conditions, metabolic functions would not be affected, as the ergosterol level was not dramatically reduced below the level required to fulfil the metabolic function, as published for *S. cerevisiae*.[7,11,43] So $\Delta^{8,14}$- and Δ^8-sterols may also not be as efficient as ergosterol for the bulk membrane function. In agreement with this, it was noticed that low concentrations of tridemorph and fenpropidin (below 2·4 µg ml^{-1}), leading to ergosterol biosynthesis inhibition without growth inhibition in dry weight terms, had effects on the morphology of the mycelium which became swollen, very branched and short compared to controls. Conidiation was also affected (data not shown). Such morphology modifications have already been observed with other SBIs and fungi.[32,49] They could be due to membrane alteration because of the incorporation of $\Delta^{8,14}$- or Δ^8-abnormal sterols, leading to hyperfluidity[12,30] and the abnormal deposition of chitin,[32,49] probably due to abnormal distribution of chitin synthase in the plasma membrane.[12,30] Several other perturbations in the cellular process would be provoked by hyperfluidity.[10–12,41,50] These

modifications might further lead to fungitoxicity, especially when abnormal sterol accumulation and ergosterol decrease would be significant. Our study thus indicates that ergosterol is essential for both bulk membrane and metabolic functions.

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